THE PANCREAPEDIA
Exocrine Pancreas Knowledge Base
www.pancreapedia.org

METHODS PAGE

Purification of Pancreatic Zymogen Granules using Percoll Gradients

Xuequn Chen and John A. Williams
University of Michigan, Department of Molecular & Integrative Physiology
Ann Arbor, MI 48109-0622
emails: xuequnc@umich.edu; jawillms@umich.edu

Version 1.0, January 2, 2010 [DOI: 10.3998/panc.2010.3]

Pancreatic zymogen granules (ZG) have a density between 1.13-1.15 g/ml and are suitable for purification by density gradients. Because of its ease of use, Percoll™ has been widely used to separate organelles and has been used by multiple laboratories to isolate highly purified zymogen granules (1-4, 6-10, 12-16). Percoll is non-toxic colloidal silica, supplied as a 23% (w/w) colloidal solution in water with a density of 1.130 g/ml and has a very low osmolality (<25 mOs/kg H2O). Self-generating gradients ranging from 1.0-1.3 g/ml are achievable by centrifugation using angle-head rotors. Biological particles having sedimentation coefficient values of >60S can be successfully banded on gradients of Percoll. The density gradient can be quantitated using multiple colored beads of different densities. Percoll technology has been discussed in great detail previously (11). This chapter describes a successfully implemented procedure for purifying pancreatic zymogen granule in large quantity based on Percoll gradient centrifugation and the subsequent separation of ZG membrane and content. A modification is also presented for purifying smaller amounts of granules from isolated pancreatic acini.

1. Materials

1.1 Isolation of ZGs

1. Typically, pancreases are obtained from Sprague Dawley rats with body weight between 250 and 300 g.
2. Homogenization buffer: 0.25 M sucrose, 25 mM MES (2-Morpholinoethanesulfoni acid, monohydrate) pH 6.0 (adjusted with 1N HCl), 0.1 mM $MgSO_4$, 2 mM EGTA and 0.1 mM phenylmethylsulfonyl fluoride (*see* **Note 1**). It is made from stock solutions (2 M sucrose and 1 M MES, pH 6.0) stored at 4°C.
3. Medium sized Teflon glass homogenizer. We use a type B from Thomas Scientific, (Swedesboro, NJ) which has a serrated Teflon pestle and a clearance between 0.13-0.18 mm. Another source for homogenizers is Kimble/Kontes (Vineland, NJ)
4. Percoll™ (Amersham Biosciences).
5. Tabletop or other refrigerated preparative centrifuge.
6. Beckman ultracentrifuge with a Ti 70.1 rotor

(other high speed angle rotors can be used although the angle will affect the shape of the gradient). Open topped thick walled polycarbonate ultracentrifuge tubes for this rotor are, 10 mL, 16 x 76 mm, from Beckman.

1.2 Purification of ZG membranes

1. Nigericin (Sigma) is dissolved in ethanol at a concentration of 10 mg/mL and stored at 4 °C.
2. Protease inhibitor cocktail (Roche) is used according to company instruction.
3. ZG lysis buffer: 150 mM sodium acetate, 10 mM MOPS, pH 7.0, 27 μg/ml Nigericin, 0.1 mM MgSO4, 0.1 mM phenylmethylsulfonyl fluoride supplemented with protease inhibitors cocktail. The stock solutions of 1 M sodium acetate and 1 M MOPS, pH 7.0 (adjusted with 1N HCl), are stored at 4 °C.
4. Beckman ultracentrifuge with a Ti 70.1 rotor.
5. 250 mM KBr.
6. 0.1 M Na_2CO_3, pH 11.0. (adjusted with 1N HCl)

2. Methods

2.1 Isolation of ZGs

1. In a typical experiment to prepare a significant number of granules or to isolate the ZG membrane, 10-12 rats are sacrificed by decapitation following CO_2 anesthesia. The blood is drained (*see* **Note 2**) and the pancreases are removed, minced with a small scissors and homogenized (two pancreases at a time) in 10 ml of ice-cold homogenization buffer. The homogenization is conducted for 9 strokes using a 30ml Teflon glass homogenizer driven by a heavy duty wall mounted electric motor. If only ZG are needed, the number of rats can be reduced.
2. Homogenates are combined and distributed in six 15-ml tubes and then centrifuged first at 300 g for 10 min at 4 °C to remove unbroken cells and nuclei and the supernatant is transferred to six new tubes and centrifuged at 2,000 g for 10 min to generate a white particulate enriched in ZGs covered by a tan layer containing mainly mitochondria (*see* **Note 3**).
3. The particulate is gently resuspended in 40 mL of homogenization buffer and mixed with 60 mL of Percoll solution containing 50 mL of Percoll, 7.5 mL of 2 M sucrose and 600 μL of 1 M MES, pH 6.0 (*see* **Note 4**).
4. The mixture is distributed in ten polycarbonate ultracentrifugation tubes and centrifuged in a Beckman ultracentrifuge at 30,000 rpm (60,000 g) for 20 min using a Ti 70.1 rotor.
5. The dense white ZG bands near the bottom of the centrifuge tube are collected with a plastic pipette and diluted in 60 mL of homogenization buffer. In order to remove excess Percoll, this suspension is centrifuged to pellet ZGs at 1,000 g for 10 min. This pellet normally contains about 20 mg of protein. If further purification is necessary, the ZG band can be subjected to a second Percoll gradient or the ZG pellet obtained from the Percoll gradient can be washed multiple times.
6. The high purity of the preparation has been evaluated by electron microscopy in previous studies (2, 8, 16) and also validated by the enrichment of digestive enzyme activity, usually 4-5 fold enrichment in the ZG preparation compared to total homogenate and the reduced content of mitochondrial or microsomal enzymes (2, 8). The suspension of granules can also be spread out on a glass slide and immunostained for granule membrane markers such as Rab3D in which case essentially all visualized particles stain (7).

2.2 Separation of ZG membranes and content

1. To purify ZG membrane, the above ZG pellets are resuspended in 20 mL of ZG lysis buffer and incubated at 37 °C for 15 min. The

lysate, which becomes clear at the end of the incubation, is centrifuged at 38,000 rpm (100,000 g) for one hour in a Beckman ultracentrifuge using a Ti 70.1 rotor to pellet the ZG membrane. The supernatant is saved as ZG content and contains about 95-98% of the protein. If desired the protein can be concentrated by acetone or TCA precipitation or fluid removed by ultrafiltration.

2. To remove absorbed content proteins from the ZG membranes, the pellet is re-suspended by pipeting thoroughly in 10 ml of 250 mM KBr and recentrifuged. To further purify the membrane and remove some of the peripheral membrane proteins, the ZG membrane pellet is resuspended in 0.1 M Na_2CO_3 (pH 11.0) and incubated on ice for 30 min, then centrifuged for one hour at 38,000 rpm (*see* **Notes 5, 6**).

3. In a typical experiment using 10-12 rats, normally about 1 mg and 300 μg of proteins can be recovered from ZG membrane before and after the KBr and Na_2CO_3 washes respectively (*see* **Note 7**). The protein content of the ZGM can be evaluated by Western blotting or mass spectrometry.

2.3 Micropurification of ZG from isolated pancreatic acini

A smaller scale version has been used to isolate granules from 1-2 mouse pancreas or from isolated acini when it is desired to evaluate the effect of secretagogue stimulation (5).

1. Isolated pancreatic acini are prepared by collagenase digestion of 1-2 mouse pancreas. Following incubation they are rinsed with cold PBS and subdivided into up to 4 fractions. Then each re-suspended in 1.5 ml homogenization buffer and homogenized in a Kontes Teflon-glass homogenizer (size 20) at 900 rpm for 6 strokes.

2. Transfer the homogenate to 1.5ml Eppendorf tubes and centrifuge at 200 x g for 10 min at 4 °C. Then take supernatant and spin at 1500 x g at 4 °C for 10 min to obtain a crude granule fraction.

3. Remove supernatant and gently re-suspend the pellet with 1.2 ml, 50% Percoll and transfer to 2ml centrifuge tubes from Beckman for ultracentrifugation

4. Centrifuge at 38,000 rpm (67,000 g) for 20 min at 4 °C in a Beckman TLX Optima ultracentrifuge using a TLA 45 rotor

5. Collect bottom white bands from the tubes and wash each with 1.5 ml homogenization buffer at 1500 x g or 10 min 4 °C to remove Percoll.

3. Notes

1. Because phenylmethylsulfonyl fluoride is not stable, it is added to the buffer from a 0.2 M stock solution stored at -20 °C immediately before use and every hour thereafter.

2. The blood needs to be drained as completely as possible. Large amount of residual blood in pancreas tissue may contaminate ZG preparation and is apparent as a reddish pellet at the very bottom of the white pellet after the second low-speed centrifugation. This blood contaminant is hard to efficiently separate by Percoll gradient centrifugation.

3. The loose layer of tan pellet containing mainly mitochondria can be largely removed by rinsing the pellet with homogenization buffer several times. The residual mitochondria appear in the top band of the centrifuge tubes after Percoll gradient ultracentrifugation.

4. In order to use Percoll to prepare a gradient, the osmolality of Percoll needs to be adjusted with sucrose to make Percoll isotonic. Ideally this can be done by dialyzing the Percoll solution against buffered sucrose solution. However, for most work this is not necessary. However, the fact that Percoll beads occupy part of the suspension volume

needs to be taken into consideration (5).

5. The high salt wash alone is not efficient to remove absorbed ZG content proteins**.** In Coomassie stained 2D gels of 300 μg of KBr-washed ZGM proteins, only predominant membrane proteins such as GP2 and dipeptidase and contaminant content proteins could be detected. In contrast, small GTPase such as Rab27B and Rab11 became apparent in Coomassie stained 2D gels of 300 μg of Na_2CO_3-washed ZGM proteins.

6. ZG membrane pellets can be used immediately after preparation or alternatively stored in the centrifuge tubes at -80 °C with the caps sealed with parafilm.

7. The procedure of purifying ZGs using Percoll gradient has been scaled up accordingly to purify ZGs from bovine (14) or pig (10) pancreas using a larger centrifuge rotor.

References

1. **Anderie I, Blum R, Haase W, Grinstein S, and Thevenod F.** Expression of NHE1 and NHE4 in rat pancreatic zymogen granule membranes. *Biochem Biophys Res Commun* 246: 330-336, 1998. PMID: 9610358

2. **Burnham DB, Munowitz P, Thorn N, and Williams JA.** Protein kinase activity associated with pancreatic zymogen granules. *Biochem J* 227: 743-751, 1985. PMID: 4004796

3. **Chen X and Andrews PC.** Purification and proteomics analysis of pancreatic zymogen granule membranes. *Methods Mol Biol* 432: 275-287, 2008.
PMID: 18370025

4. **Chen X and Andrews PC.** Quantitative proteomics analysis of pancreatic zymogen granule membrane proteins. *Methods Mol Biol* 528: 327-338, 2009.
PMID: 19153703

5. **Chen X, Ernst SA, and Williams JA.** Dominant negative Rab3D mutants reduce GTP-bound endogenous Rab3D in pancreatic acini. *J Biol Chem* 278: 50053-50060, 2003. PMID: 14522985

6. **Chen X, Ulintz PJ, Simon ES, Williams JA, and Andrews PC.** Global topology analysis of pancreatic zymogen granule membrane proteins. *Mol Cell Proteomics* 7: 2323-2336, 2008. PMID: 18682380

7. **Chen X, Walker AK, Strahler JR, Simon ES, Tomanicek-Volk SL, Nelson BB, Hurley MC, Ernst SA, Williams JA, and Andrews PC.** Organellar proteomics: analysis of pancreatic zymogen granule membranes. *Mol Cell Proteomics* 5: 306-312, 2006. PMID: 16278343

8. **De Lisle RC, Schulz I, Tyrakowski T, Haase W, and Hopfer U.** Isolation of stable pancreatic zymogen granules. *Am J Physiol* 246: G411-418, 1984. PMID: 66096

9. **Gasser KW and Holda JR.** ATP-sensitive potassium transport by pancreatic secretory granule membrane. *Am J Physiol* 264: G137-142, 1993. PMID: 8430796

10. **Hofken T, Linder D, Kleene R, Goke B, and Wagner AC.** Membrane dipeptidase and glutathione are major components of pig pancreatic zymogen granules. *Exp Cell Res* 244: 481-490, 1998. PMID: 9806799

11. **Pertoft H.** Fractionation of cells and subcellular particles with Percoll. *J Biochem Biophys Methods* 44: 1-30, 2000. PMID: 10889273

12. **Rindler MJ, Xu CF, Gumper I, Smith NN, and Neubert TA.** Proteomic analysis of pancreatic zymogen granules: identification of new granule proteins. *J Proteome Res* 6: 2978-2992, 2007. PMID: 17583932

13. **Roussa E, Alper SL, and Thevenod F.** Immunolocalization of anion exchanger AE2, Na(+)/H(+) exchangers NHE1 and NHE4, and vacuolar type H(+)-ATPase in rat pancreas. *J Histochem Cytochem* 49: 463-474, 2001. PMID: 11259449

14. **Thevenod F, Haase W, and Hopfer U.** Large-scale purification of calf pancreatic zymogen granule membranes. *Anal Biochem* 202: 54-60, 1992. PMID: 1377890

15. **Wagner AC, Wishart MJ, Mulders SM, Blevins PM, Andrews PC, Lowe AW, and Williams JA.** GP-3, a newly characterized glycoprotein on the inner surface of the zymogen granule membrane, undergoes regulated secretion. *J Biol Chem* 269: 9099-9104, 1994. PMID: 8132647

16. **Yule DI, Ernst SA, Ohnishi H, and Wojcikiewicz RJ.** Evidence that zymogen granules are not a physiologically relevant calcium pool. Defining the distribution of inositol 1,4,5-trisphosphate receptors in pancreatic acinar cells. *J Biol Chem* 272: 9093-9098, 1997. PMID: 9083036